Isothermal Titration Calorimetry (ITC):

Theory and Applications
Instrumentation Basics

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Waters™
TA
Instruments

ITC Theory

Basics of an Isothermal Titration Calorimetry (ITC)

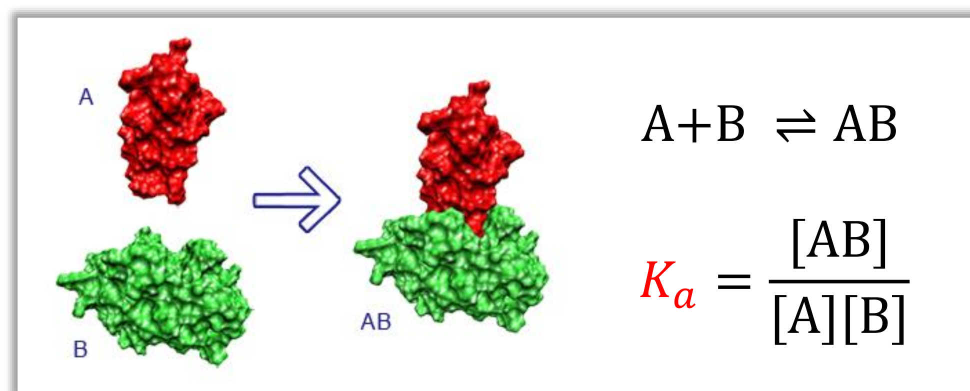
Calorimetry *is an application of the First Law of Thermodynamics to heat transfers and allow us to* ***measure*** *the* ***enthalpy*** *of a reaction including* ***binding****. It is a label free and non-optical method meaning no specific molecular groups or labels are required.*

- The signal in a calorimeter is the "heat" of a process ⇒ ΔH

  ΔH reflects the amount of heat energy required to achieve a particular state – *direct measurement*

- Gibbs Free Energy Change (ΔG): it dictates the direction of binding equilibria; $\Delta G = -RT\ln K_a$ – *direct measurement*
- We can also determine the value of ΔS because it also measures the value of K_a or ΔG.

  ΔS measure how easily this energy is distributed among various molecular energy levels – *indirect measurement*



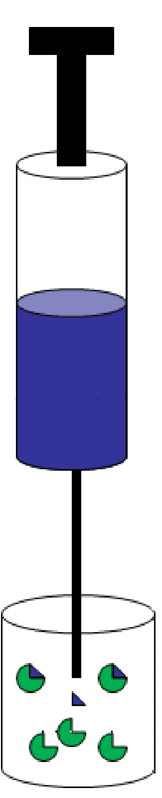
Gibbs Free Energy (ΔG) *is a balance between Enthalpy* (ΔH) *and Entropy* (ΔS)

$$\Delta G = \Delta H - T\Delta S$$

Association Constant, K_a vs Dissociation Constant, K_d: $K_d = 1/K_a$

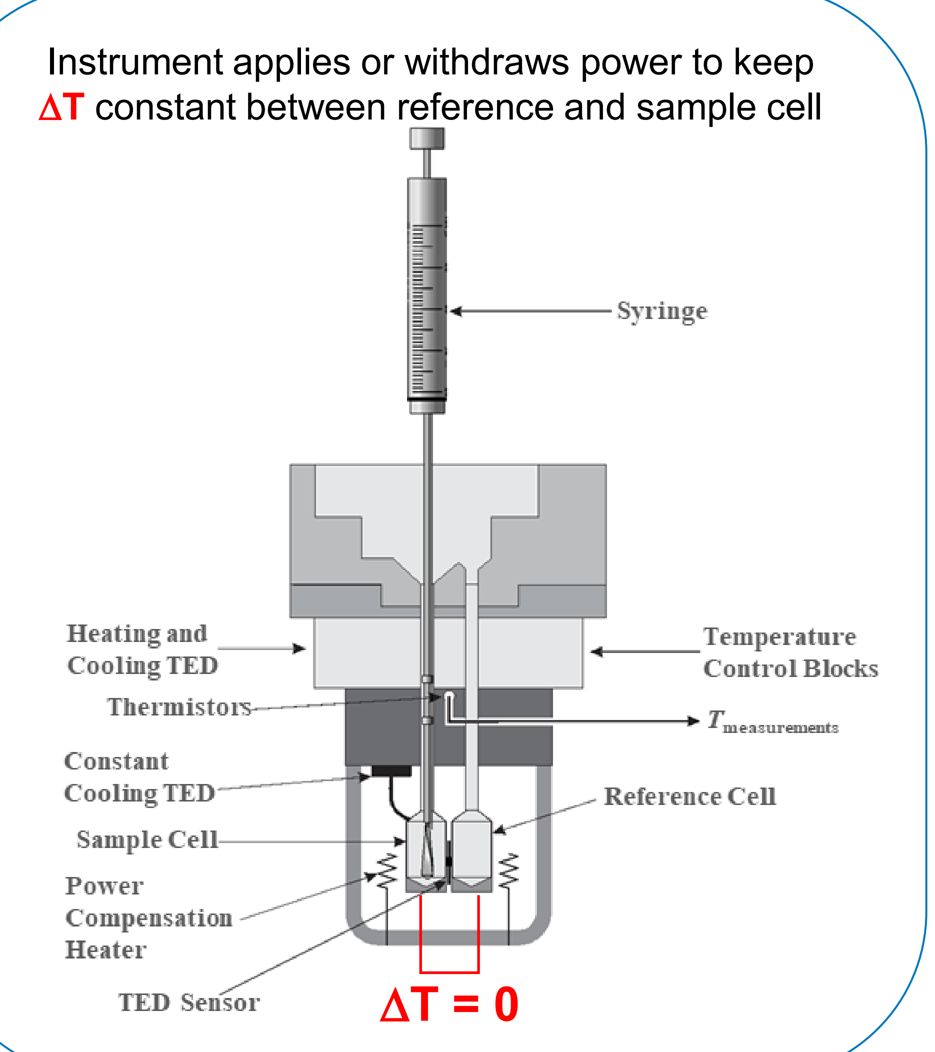
Basics of an Isothermal Titration Calorimetry (ITC) Experiment

- Mix two solutions
  - Mixing chemicals will generate heat
- Measure Heat (q)
  - Proper controls ensure certain heat events are measured
- Fit data to assumed model
  - Model based from shape of curve and/or background knowledge
- Analyze data
  - Quantify K_d, ΔG, ΔH, ΔS, n, CMC, ΔC_p, enzyme kinetics

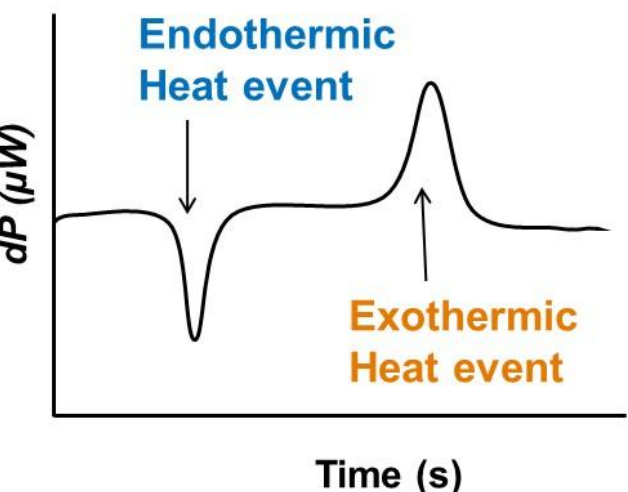


ITC Instrument: Power Compensation Calorimeter

Instrument applies or withdraws power to keep ΔT constant between reference and sample cell



Raw output is the change in power (dP, μW) with time



- As titrant is introduced to the titrand, instrument will apply or withdraw power
  - Will keep reference and sample cells at constant temperature
- Areas of the peaks are the heat (q), and at constant pressure q = ΔH

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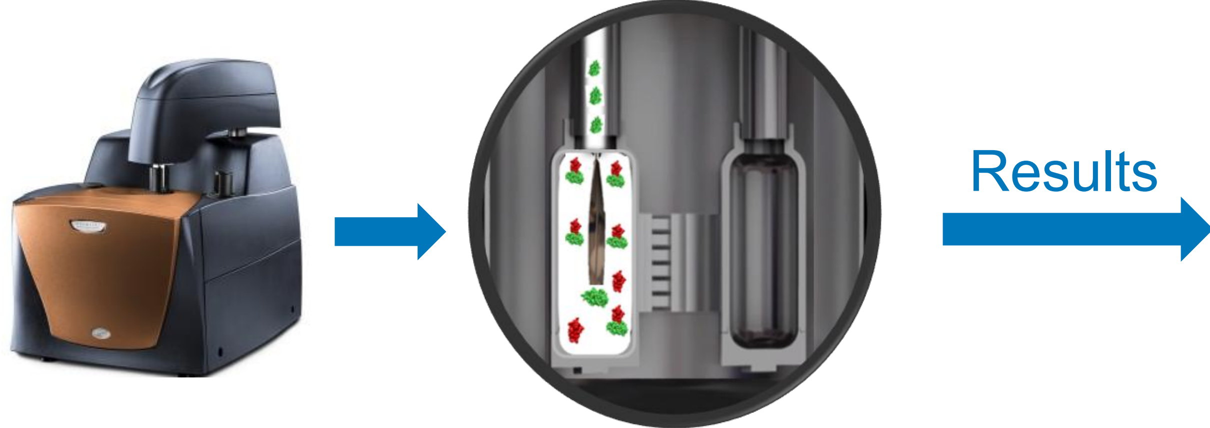
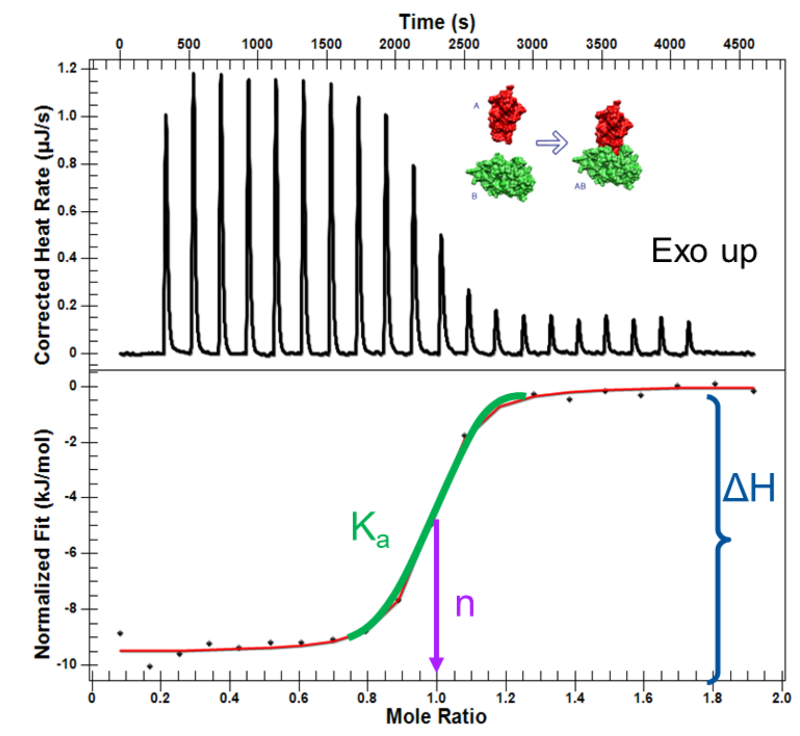
Isothermal Titration Calorimetry

- The titrant (ligand) is loaded into a syringe at 10x greater concentration than the titrand (substrate) in the cell.



- Substrate concentration is ideally in the range of 1-1000*K_d.
- Heat (q) is produced when the complex is formed. As free substrate decreases, so does the heat .



Thermodynamic parameters:

Stoichiometry, n   Binding constant, K_a,   Enthalpy, ΔH

$\Delta G = - RT\ln(K_a)$ and $\Delta G = \Delta H - T\Delta S$

Free energy, ΔG Entropy, ΔS

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Beyond K_d – Values from ITC

Quantify:

K_d, ΔH, n (stoichiometry)

*Binding constants in the order of 10^2-10^9 M^{-1} can be accurately calculated

ΔG, ΔS

ΔC_p, $\Delta[H^+]$

CMC

Rationalize:

Structural changes

Lead optimization

- Thermodynamic parameters
  - ΔG = = RTln(K)
  - ΔG = ΔH – TΔS
- Heat capacity
  - $\Delta C_p = (\partial \Delta H/\partial T)_p$
- Enzyme kinetics
  - K_m, K_{cat}
- Other experiments
  - CMC – critical micelle concentration
  - Dimer dissociation

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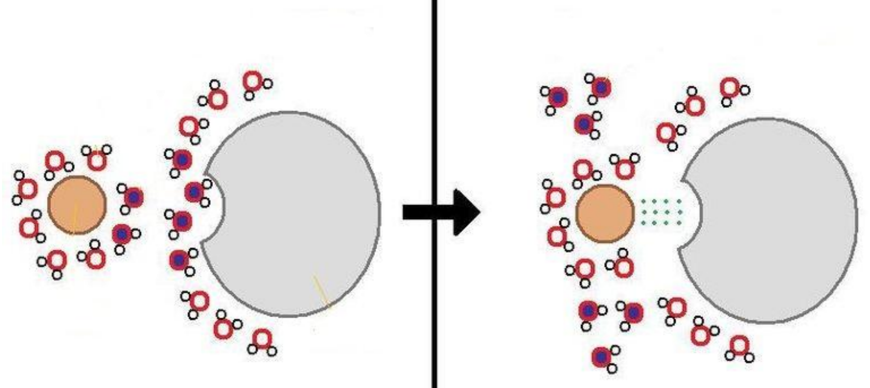
Consider the contributions to binding

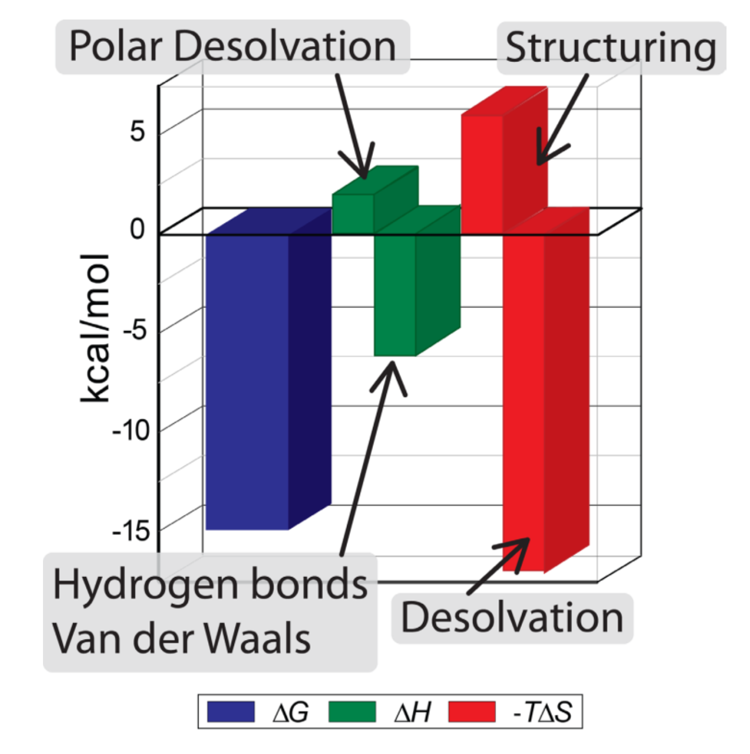


- **Enthalpy**
  - Directly associated with the strength and number of interactions broken or formed
    - Non-covalent interactions
      - Hydrogen bonds, electrostatic interactions, van der Waals forces
  - Solvent contributions are important
- **Entropy**
  - Hydrophobic interactions
  - Solvation effects (release of water molecules)

Enthalpy-Entropy Compensation

$$\Delta G = \Delta H - T\Delta S$$

$$\Delta G = -RT\ln(K_a)$$



Instrument

ITC Instruments

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Affinity ITC & Affinity ITC Auto

Fixed Cylindrical Cell

Specifications

- **Operating temperature:** 2 to 80 °C
- **Minimum Detectable Heat:** 0.04 µJ
- **Maximum Measurable Heat:** 5,000 µJ
- **Low Noise Level:** 0.0013 µW
- **Baseline Stability:** 0.02 µW/hr
- **Cell volume: 190** µL (*also* in 1.0 mL)
- **Cell geometry:** Fixed cylindrical
- **Cell material:** 24 K Gold (*also* in Hastelloy)
- **Temperature Stability:** 8 µ°C at 25 °C
- **Temperature Control:** Active heating & cooling
- **Response Time: 3.3** Seconds

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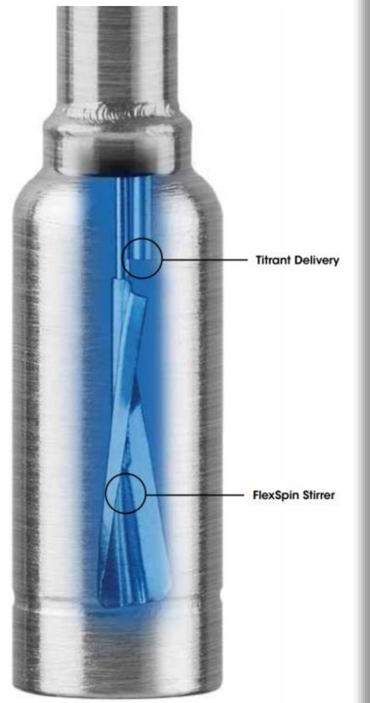
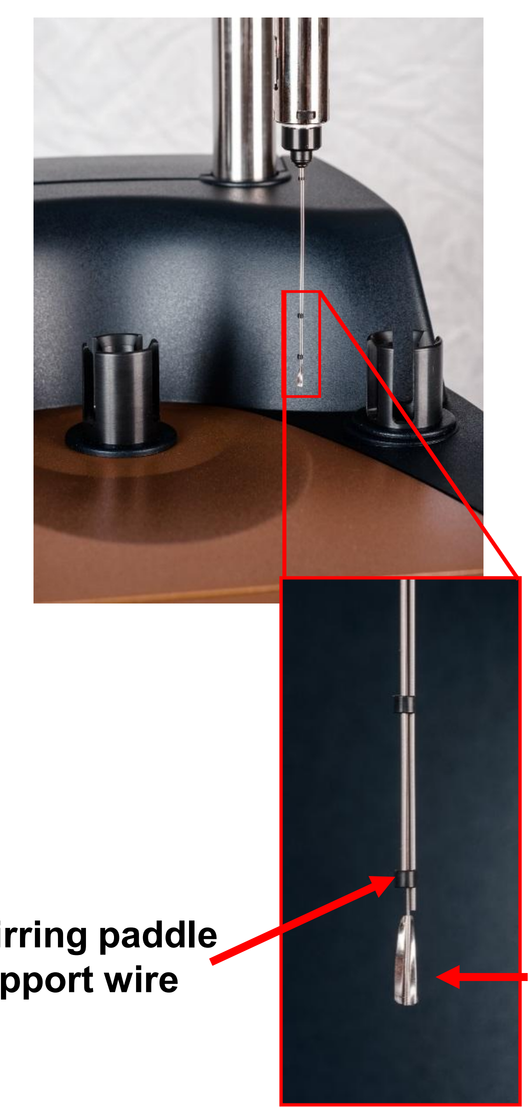
FlexSpin™ Stirring Technology





Stirring paddle support wire

New Stirring Paddle Shape

- **FlexSpin Hardware:**
  - *Thin*, *flexible* support wire
  - *Innovative* **stir paddle** design
  - *Stirring mechanism* **separate** from *injection system*
  - Easy removal and replacement
- **FlexSpin Control:**
  - Easy, intuitive on/off and stir speed settings thru ITCRun software
  - Stir speed range – 0-200 rpm
  - Recommended stir speed =125 rpm
- **FlexSpin:**
  - Sharper peaks
  - Faster return to baseline
  - Eliminates dead zones
  - Tempered steel

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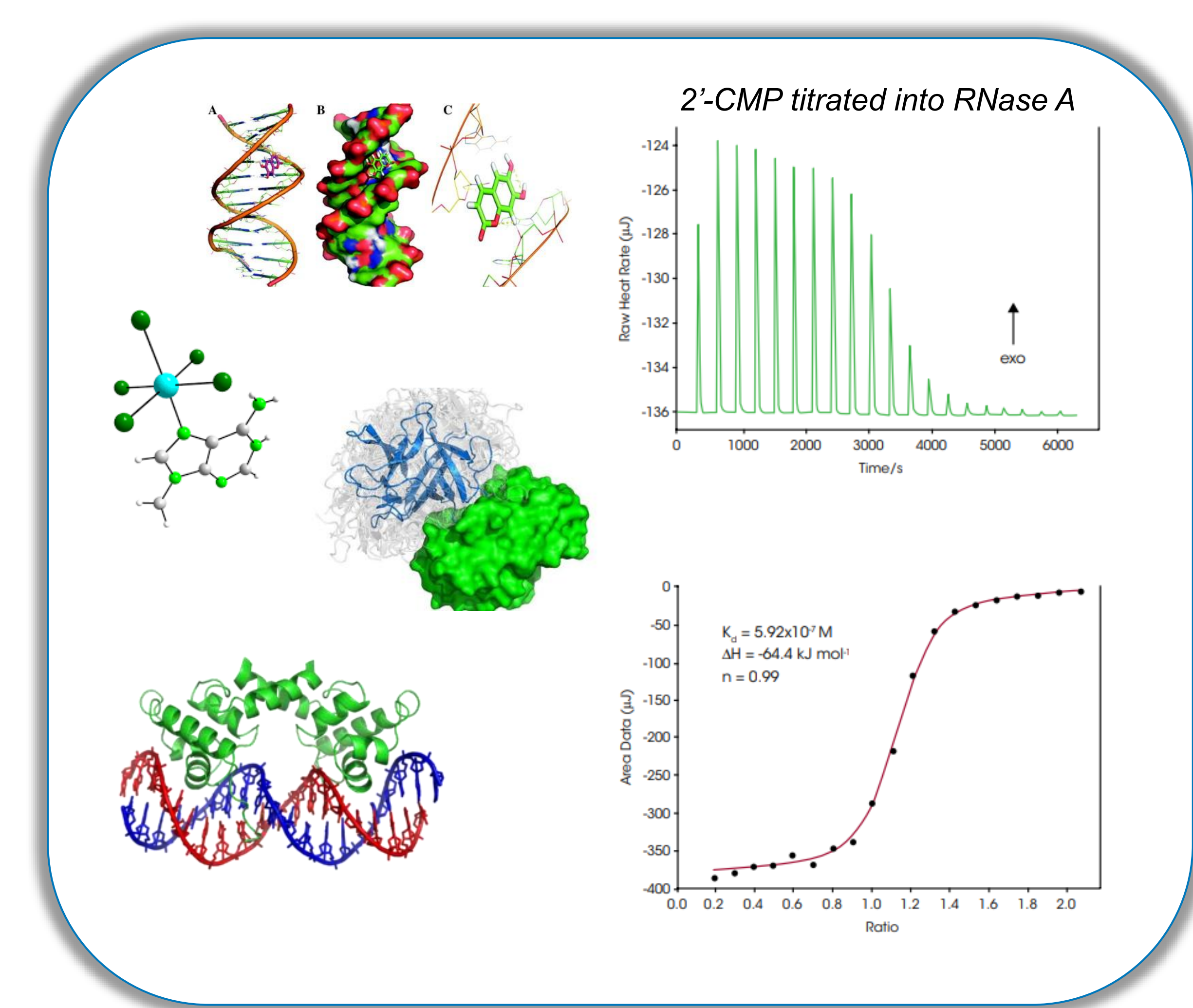
ITC Applications

ITC Applications

- Binding Interactions
- Direct or Competition
- Dimer Dissociation
- Micelle or liposome characterization
- Quality Control, [active]
- Michaelis-Menten Enzyme Kinetics



Application: Binding Interactions

Purpose: Measure the binding affinity between a protein and antibody

Method:

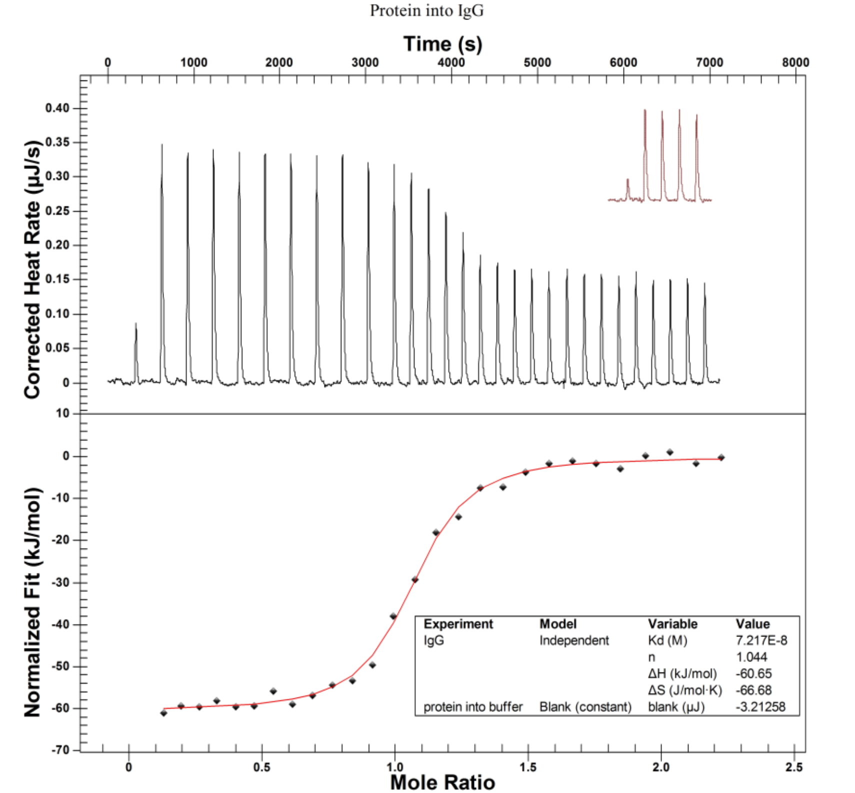
- 32 µM Protein was titrated into 8 µM IgG (black)
- 32 µM Protein was titrated into buffer (red)

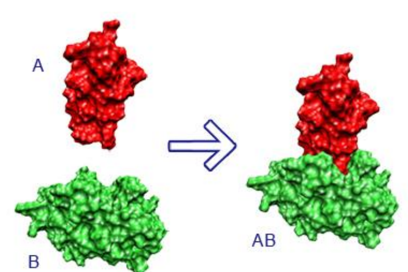
Observations:

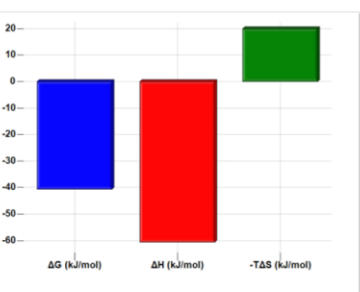
- The ITC technology generates a complete thermodynamic profile of the interaction
- Strong binding affinity (K_d = 72 nM)
- Enthalpy and stoichiometry provide additional information

ITC is a powerful analytical tool for characterizing the fundamental driving forces of molecular binding reactions

Protein-Protein Interactions







The shape of the binding curve determines the accuracy of K_a and ΔH

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Application: Binding Interactions

Purpose: Measure the binding affinity between a DNA and small molecule

Method:

- 450 µM Doxorubicin was titrated into 250 µM (bp) DNA Oligo (Blue)
- 450 µM Doxorubicin was titrated into buffer (Red)

Observations:

- Doxorubicin is one of the most important anti-cancer chemotherapeutic drugs, being widely used for the treatment of solid tumors and acute leukemias
- Average binding affinity (K_d = 1.2 µM)
- Molar ratio of 0.2 provides insight to how many base pairs the drug binds to

ITC is a powerful analytical tool for characterizing the fundamental driving forces of molecular binding reactions

Small Molecule-DNA Interactions

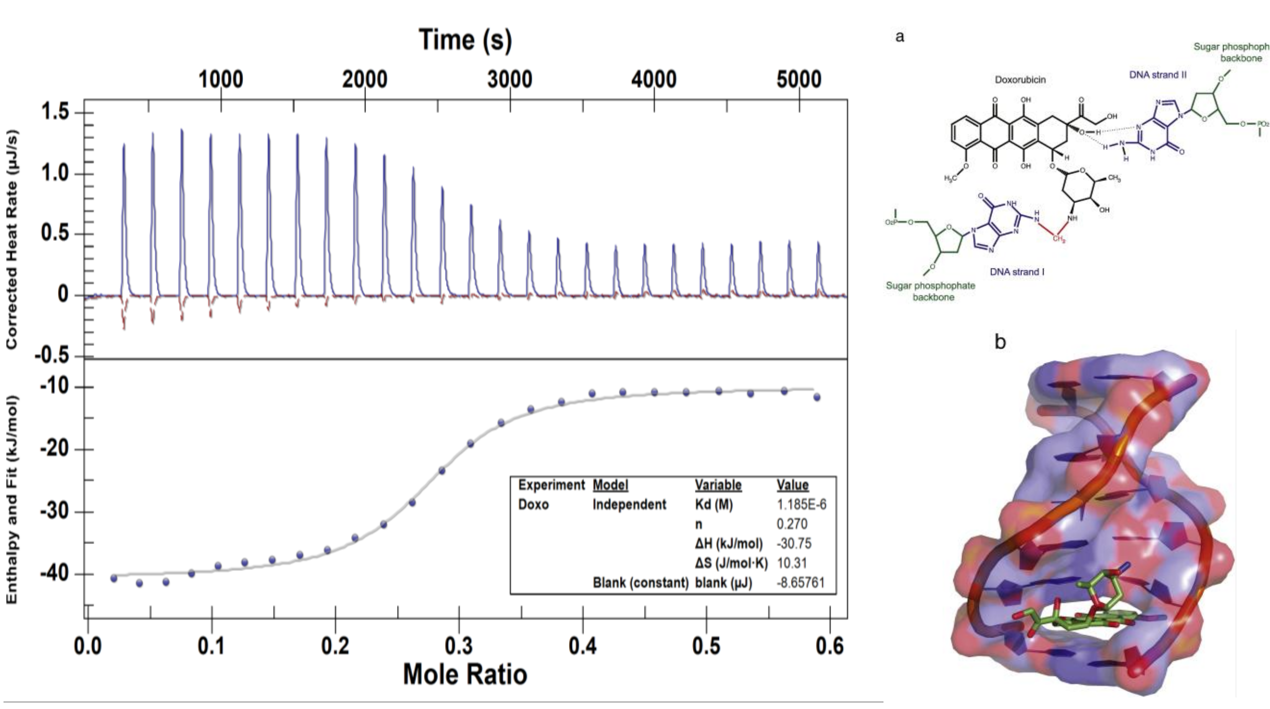


Image from: https://doi.org/10.1016/j.bbcan.2013.12.002

The shape of the binding curve determines the accuracy of K_a and ΔH

Application: Binding Interactions

Purpose: Measure the binding affinity between a ligand and enzyme

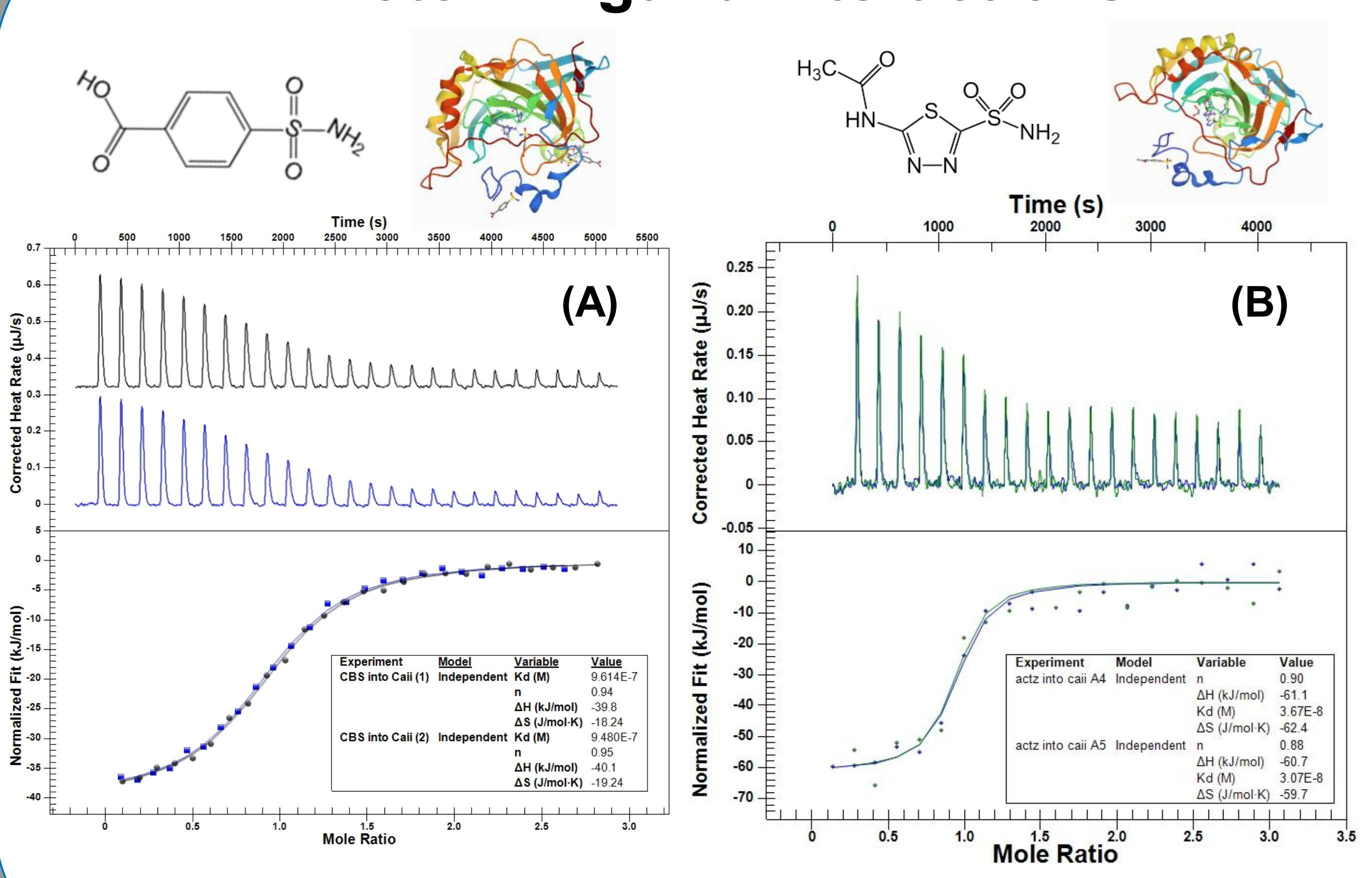
Method:

- (A) 120 µM carboxybenzenesulfonamide (CBS) was titrated into 15 µM Carbonic Anhydrase II
- (B) 30 µM acetazolamide (ACTZ) was titrated into 2.5 µM Carbonic Anhydrase II
- Run in duplicates

Observations:

- The ITC technology generates a complete thermodynamic profile of the interaction
- Different ligands bind with different strength
- Enthalpy and stoichiometry provide additional information

ITC is can be used to screen drug candidates by binding constants



The shape of the binding curve determines the accuracy of K_a and ΔH

Cartoon created from 6RFH pdb.org

Cartoon from 3HS4 pdb.org

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Application: Binding Interactions

Purpose: Measure the binding affinity between a ligand and protein

Method:

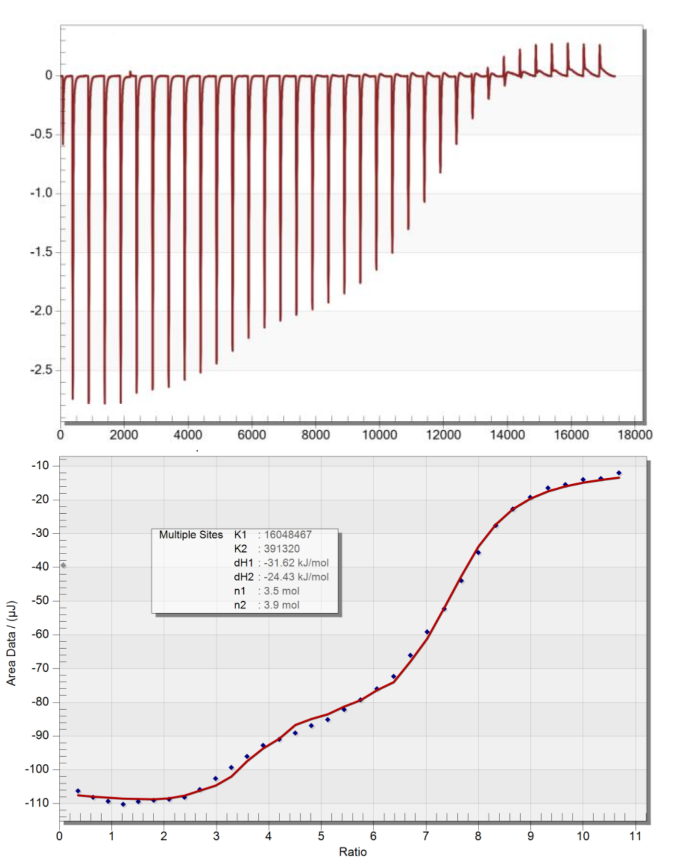
- Ligand was titrated into protein solution

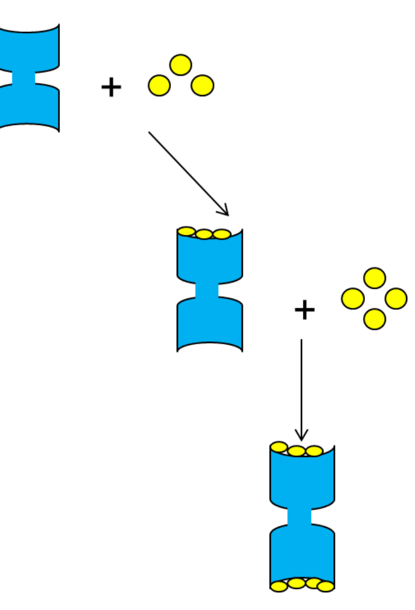
Observations:

- The ligand binds two chemically distinct sites on a receptor molecule.
- Binding isotherm for this type of interaction can have two inflections as shown in figure

The shape of the curve depends on the affinities of the ligand.

Protein-Ligand: Multiple Binding Sites





The shape of the binding curve suggests multiple binding sites

Application: Binding Interactions

Purpose: Measure the energetics of dissociation of bovine insulin in aqueous solution

Method:

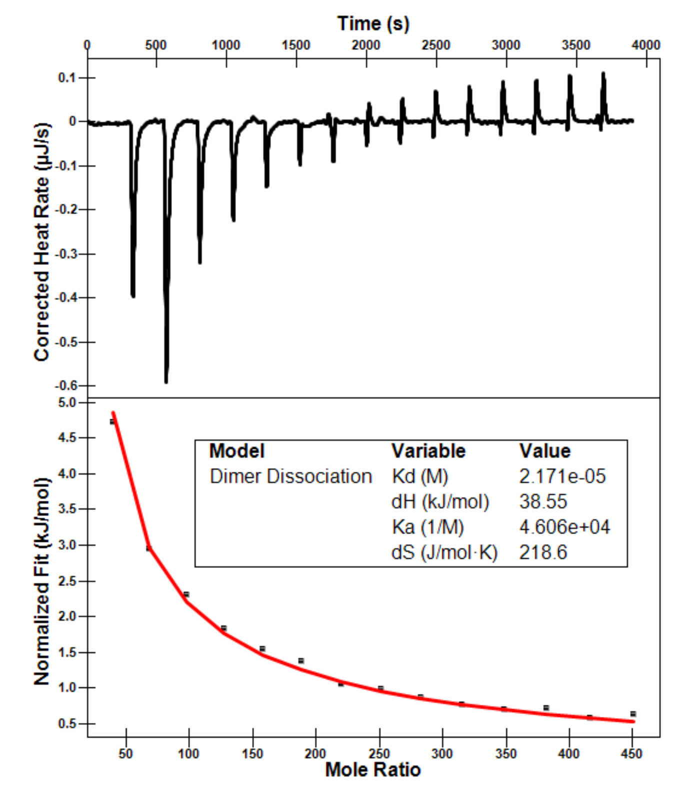
- A concentrated solution of protein is injected into buffer in the cell

Observations:

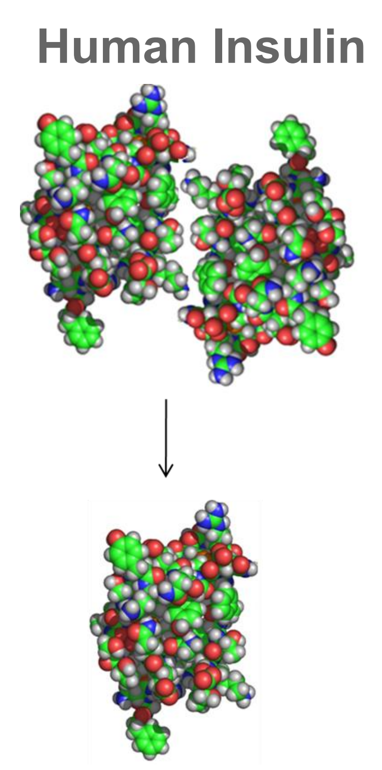
- Initially, dimer dissociates when it enters the cell, since its concentration in the cell is low.
- As its concentration increases, the dimers dissociate less, until eventually it reaches a concentration that drives dimer formation.
- The dimer dissociation model is used to study dissociation events. K_d in this model represents the dissociation constant: K_d = 22 μM

ITC is also suited for studying the dissociation of homodimers

Dimer Dissociation



Model	Variable	Value
Dimer Dissociation	Kd (M)	2.171e-05
	dH (kJ/mol)	38.55
	Ka (1/M)	4.606e+04
	dS (J/mol·K)	218.6



Data is consistent with the ITC literature values for bovine insulin collected at pH 2.5

Lovatt, Cooper, Camilleri, Eur. Biophys. J. (1996) 24:354-357

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Application: Binding Interactions

Purpose: Critical Micelle Concentration (CMC)

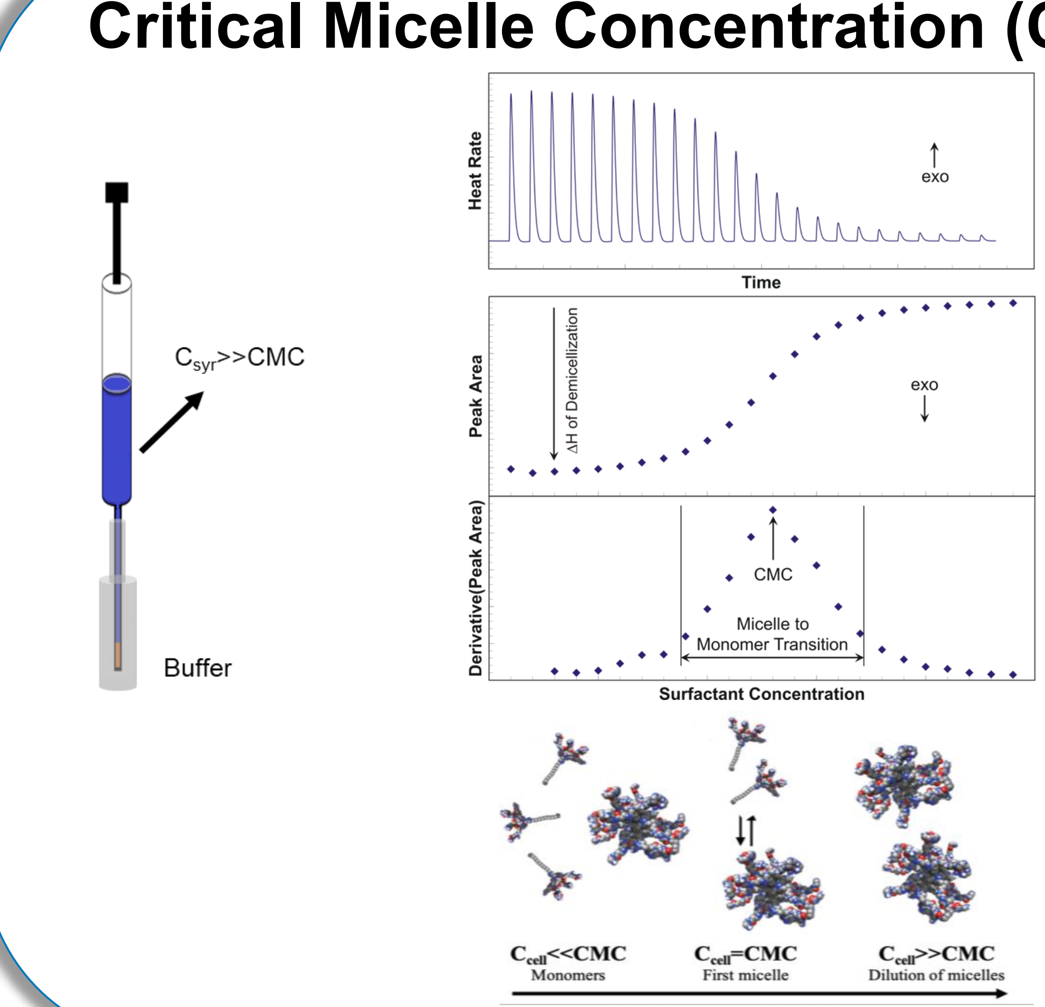
Method:

- Titrate concentrated detergent suspension (micelles) into buffer.

Observations:

- Critical micelle concentration (CMC) is the concentration at which detergents aggregate to form micelles.
- Initially micelles dissociate in sample cell. At CMC, detergent in the sample cell aggregates. Midpoint of the inflection is the CMC.

ITC can provide a comprehensive description of surfactant association



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Application: Binding Interactions

Purpose: Evaluate enzyme kinetics under steady-state conditions

Method:

- Fill syringe with excess substrate, the amount added at the end of the titration will need to be greater than the K_m.
  $[S]_{cell\ final} > K_m$
- Fill cell with a limiting amount of enzyme.

Observations:

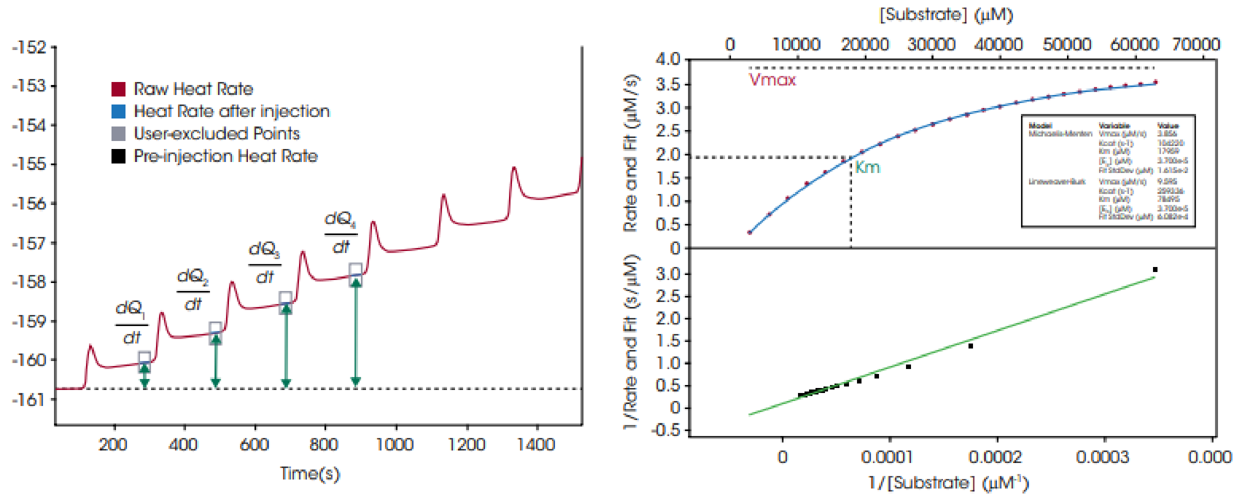
- Substrate concentration-dependent heat flow of enzymatic reactions may be used for kinetics analysis and determination of Michaelis-Menten reaction parameters
- The plateau is related to the maximum turnover, or v_{max}. Additional substrate does not increase turnover at this point, the enzyme is working at its maximum capacity.

ITC is a facile technique for characterizing enzyme kineticsparameters

Enzyme Kinetics: Multiple Injection Method

Rate of heat flow and reaction enthalpy

Michaelis-Menten and Lineweaver-Burk plots



Multiple injections of substrate into enzyme allows for multiple rate determinations under steady-state conditions within one experiment

Application: Quality Control

Purpose: Investigate if biotherapeutic is still active?

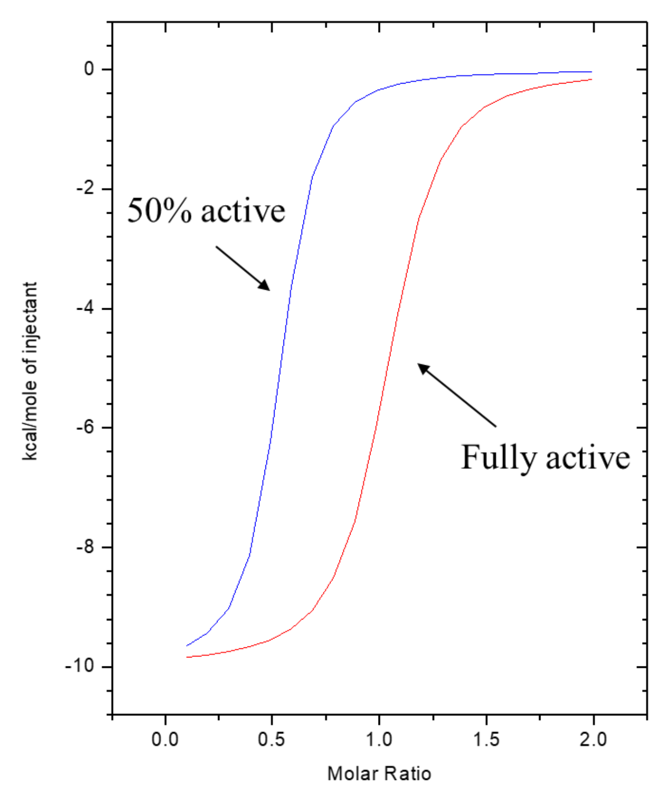
Method:
- Titrate known ligand solution into biotherapeutic solution

Observations:
- Check for unexpected stoichiometry

Uses:
- Biotherapeutic quality
- This technique is currently used by customers to test antibody batches and the effects of immobilization

ITC can be a useful tool in evaluating loss of activity in biotherapeutics

Anti-quinidine antibody batches compared



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NanoDSC Theory and Instrument

Why use a Nano DSC?

- DSC is the only technique that allows the direct measure of T_m, DC_p and DH.
  - DSC allows for calculation of entropy (DS) and free energy (DG).
- Does not require nitrogen purge
  - i.e. CD-spectroscopy
- Equally useful for macromolecules and small molecules (no MW limit)
- Compatible with essentially any buffer or additive
- Requires small sample concentrations and volume

Nano DSC Specifications and Configurations

Temp Range	-10° C to 130° C Capillary -10° C to 160° C Cylindrical
Pressure Perturbation	To 6 atm
Cell volume	0.3 mL
Cell material	Platinum – Capillary Gold – Cylindrical

Capillary can also be automated

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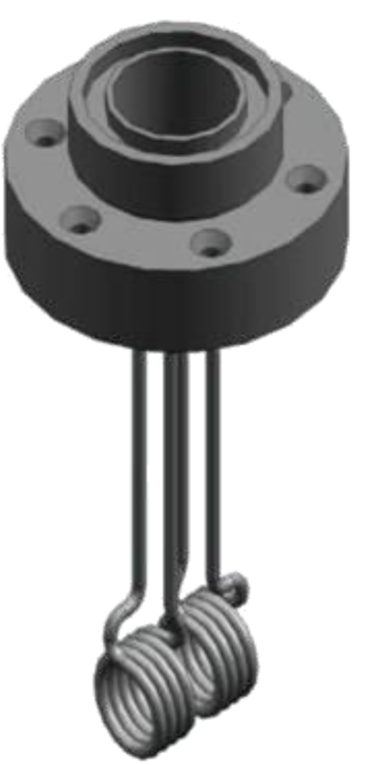
Nano DSC Cell Geometry

Continuous Capillary (130°) Fixed-cell for maximum sensitivity

- Cell Construction;
  Inert to biomaterials
  99.99% Platinum
- Small Sample Volume
  (0.3 mL)
- Attenuates or delays onset of aggregation until after protein has unfolded
- Easy-to-fill and clean design



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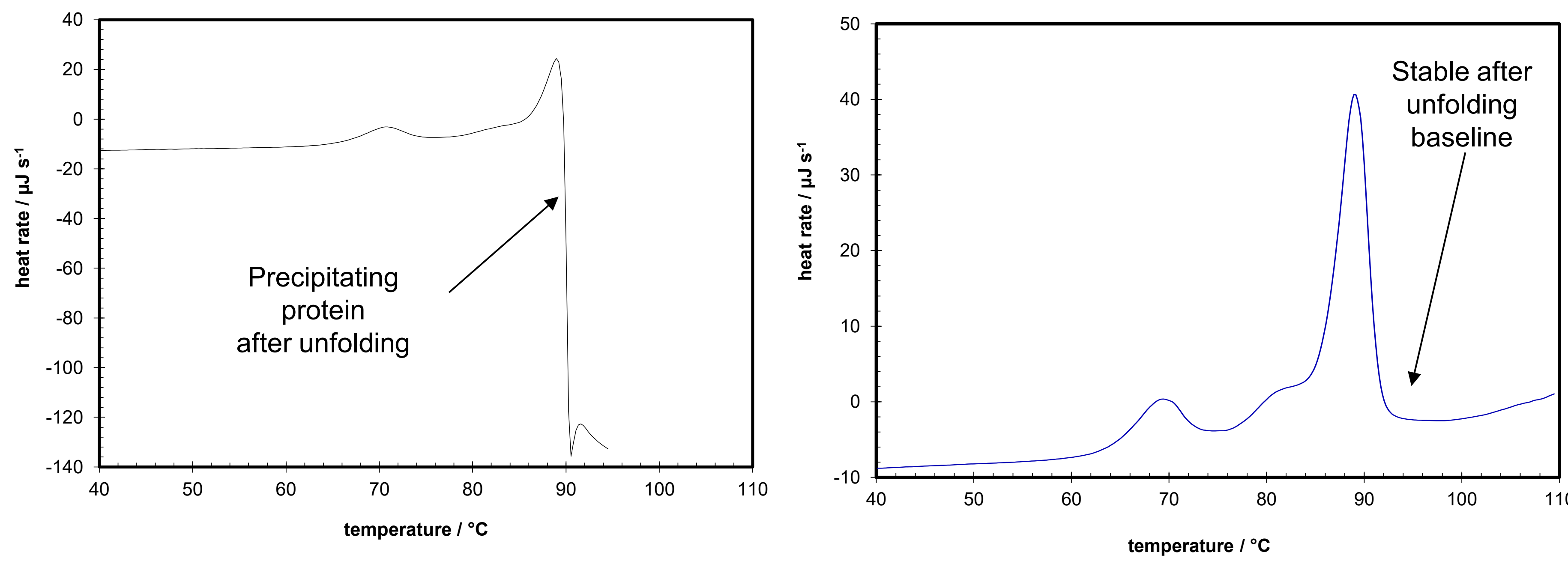
Benefit of Capillary Sample Cell

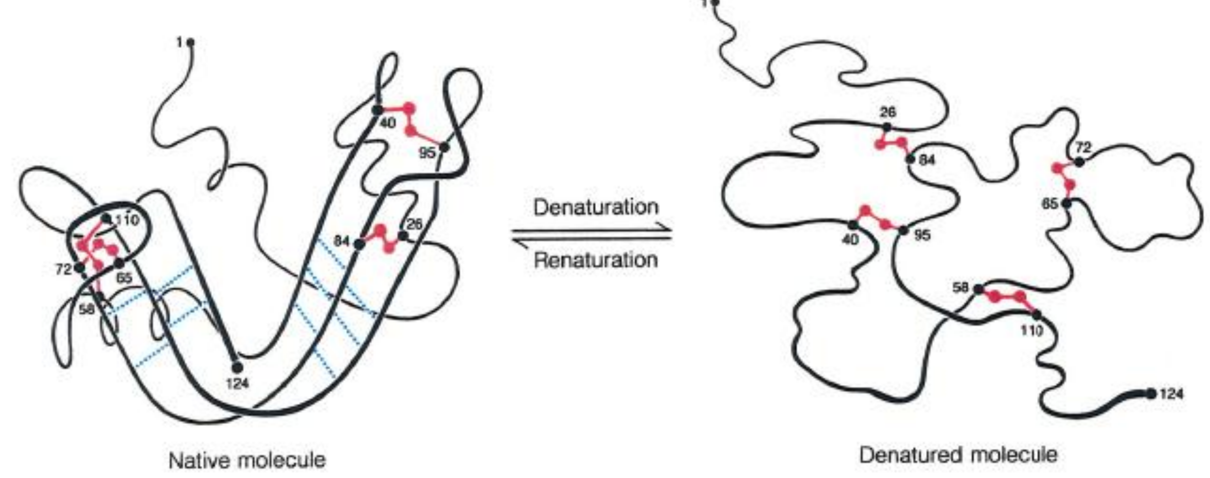
Data obtained with a DSC with "Coin and Cylindrical " shaped sample cell

Data obtained with a Nano DSC with continuous Capillary sample cell



Purified human IgG_1 monoclonal antibody in physiological buffer; 0.5 mg/mL

Two state model of protein unfolding



Enthalpically Favorable
Hydrophobic & electrostatic interactions, H^+ bonding

Entropically Favorable
Changes in solvation & conformational freedom

- Heat associated with unfolding (endothermic) and folding (exothermic) is easily measured by calorimetry, allowing thermodynamic analysis of the folding/unfolding process.
- Folding and unfolding of a small protein, a domain, or a subunit, is 'cooperative' (once started, it goes to completion).
- These small units can fold and unfold reversibly. Reversibility is directly measurable using DSC.

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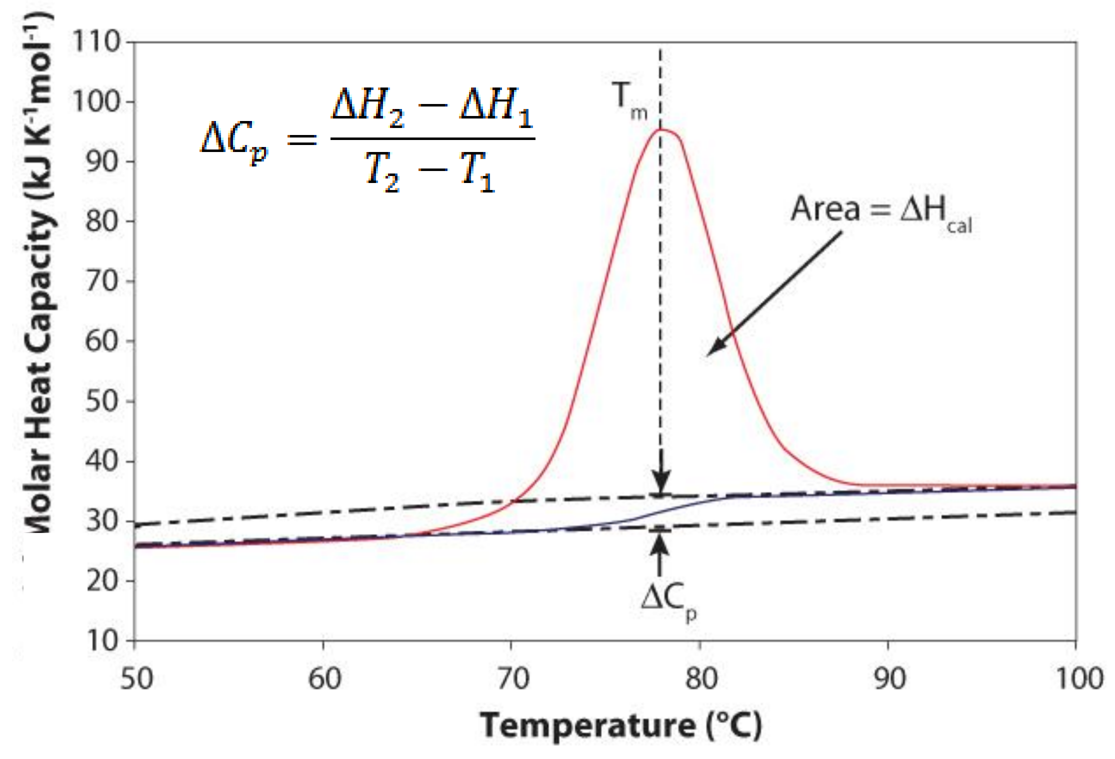
Information from DSC

- **Heat capacity change (ΔC_p)**
  - Trend: Protein with lower ΔC_p - more rigid and protein with higher ΔC_p - more flexible.
  - Primarily reflects exposure of hydrophobic groups. ΔC_p is positive.
- **Enthalpy**
- ΔH: e^2 non-covalent interactions – hydrophobic & electrostatic, H+ bonding
- **T_m**
  - Indicates macromolecular stability
  - From these measured values it is possible to calculate the entropy (ΔS) and free energy (ΔG)



ASTM E2603-08 - for verification of enthalpy and temperature of a fixed-cell DSC (www.astm.org)

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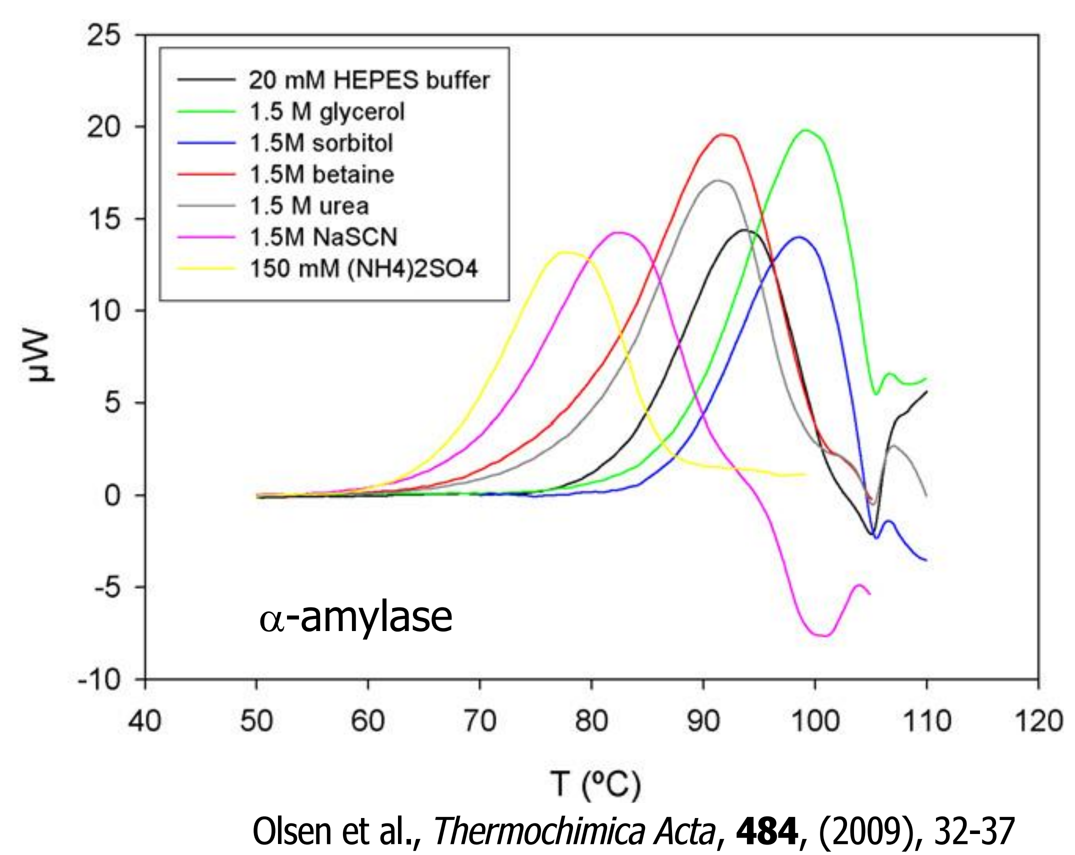
NanoDSC Applications

Applications for DSC

- Determine thermal transition (melting) temperatures
- Measure ΔH of denaturation
- Measure reversibility of thermal processes
- Measure ΔC_p of unfolding
- Determine stability macromolecules
- Measure high affinity binding (up to 10^{20} M^{-1})
- Investigate a complex milieu

Effect of additives and formulations

- Excipient to stabilize against chemical and physical degradation
- Choice of an additive or a formulation is generally determined empirically
- DSC is the fastest way of evaluating additives effect on T_m, reversibility

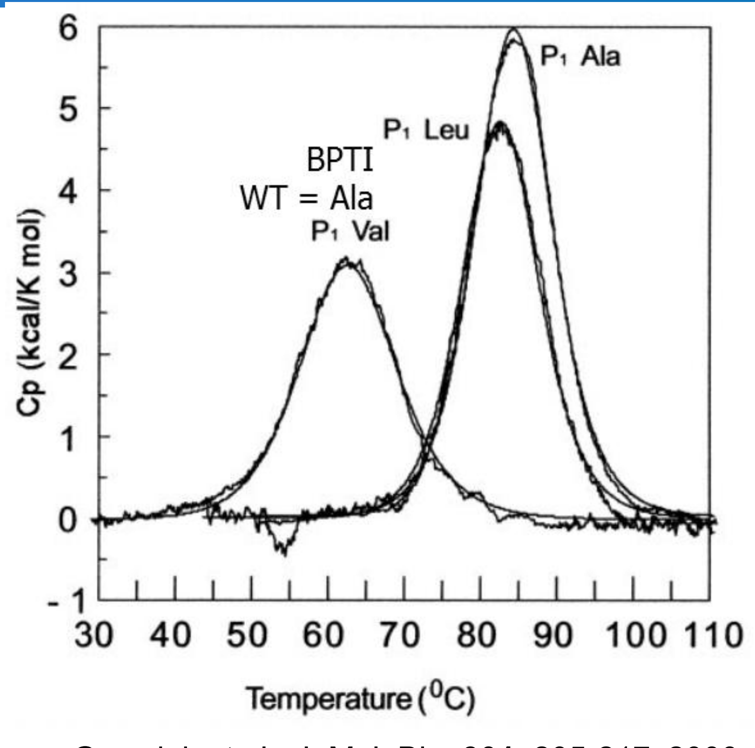


Olsen et al., *Thermochimica Acta*, **484**, (2009), 32-37

Many users stop data analysis after reporting a T_{max}, the following slides will discuss what is being missed.

Macromolecular Stability

- Mutate proteins: more stable, more specific, faster, new properties, etc.
- Useful to predict outcome of a mutation, so need a database of thermodynamically characterized proteins
- Complicated network of interactions
  - Example: enthalpic changes (changes in hydrophobic interactions, hydrogen bonding, electrostatic interactions) are compensated by entropic changes (changes in solvation, conformational freedom)



Grzesiak et al., J. Mol. Bio. **301**, 205-217, 2000

- Since $\Delta G = \Delta H - T\Delta S$, unfolding occurs when $T\Delta S$ increases sufficiently (e.g. by absorbing heat) to overcome stabilizing enthalpic interactions
- Biopolymer unfolding is endothermic

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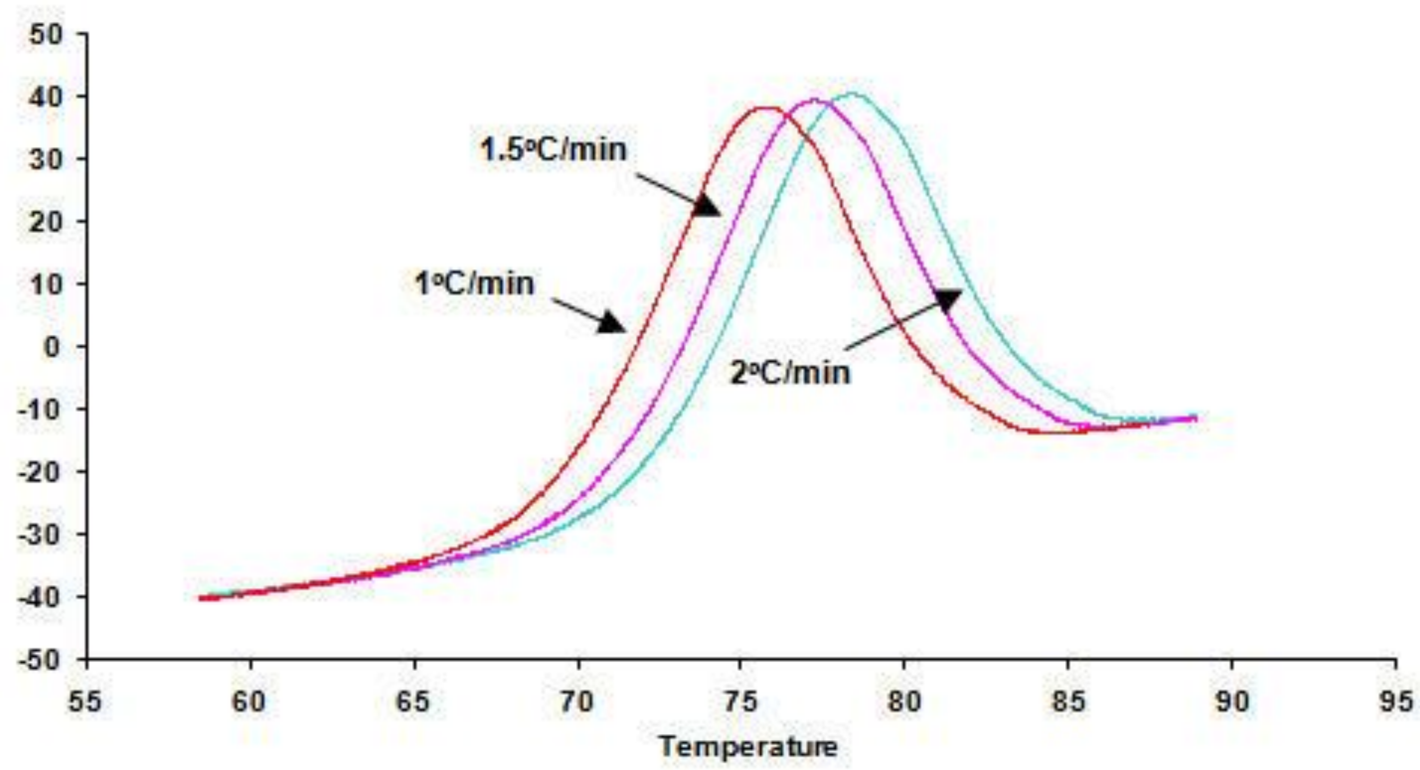
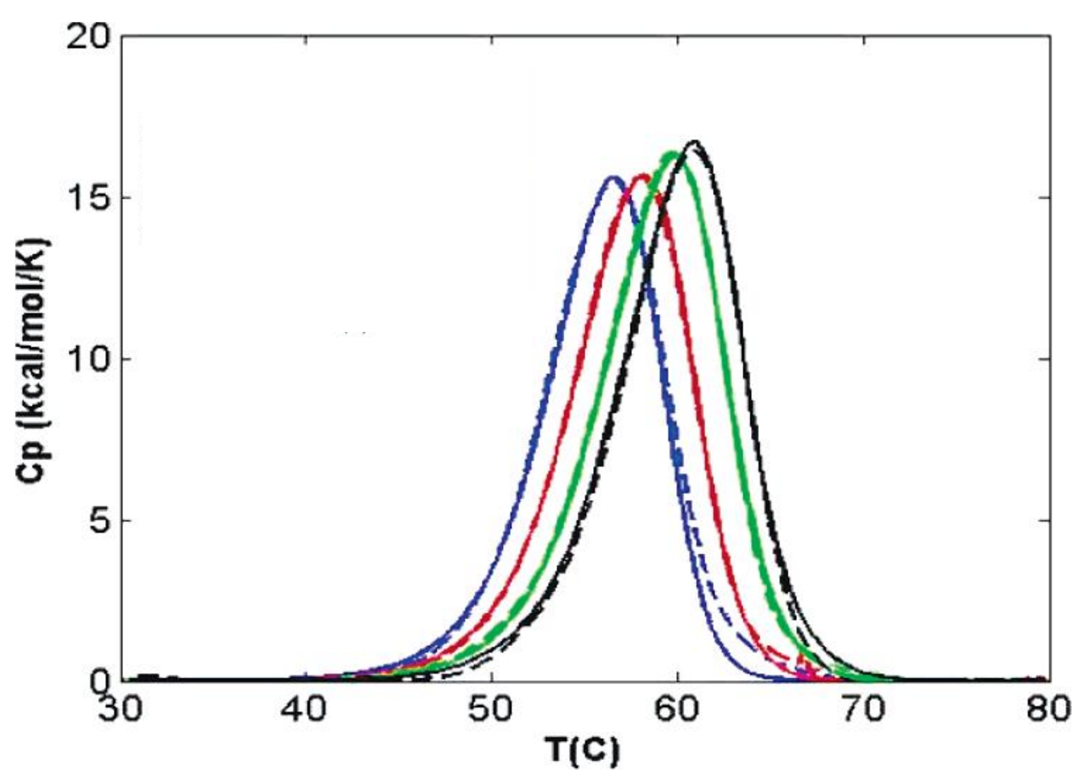
How to determine if unfolding is kinetically controlled?



- Scan rate dependence of T_m indicates that N and U are not in equilibrium.
- Their concentrations change at a rate equal to the sum of the unfolding and refolding reactions. Increasing temperature faster than system responds distorts T_m and shape.

Protein unfolding/refolding reversibility - scan rate



Remmele et al., *JACS* **127**, (2005), 8328-8339

- Interleukin-1 receptor scanned at 0.25, 0.5, 1.0 and 1.5 ^{o}C/min:
  - Scan-rate dependence of T_m indicates folded and unfolded protein are not in equilibrium
  - Unfolding is kinetically controlled

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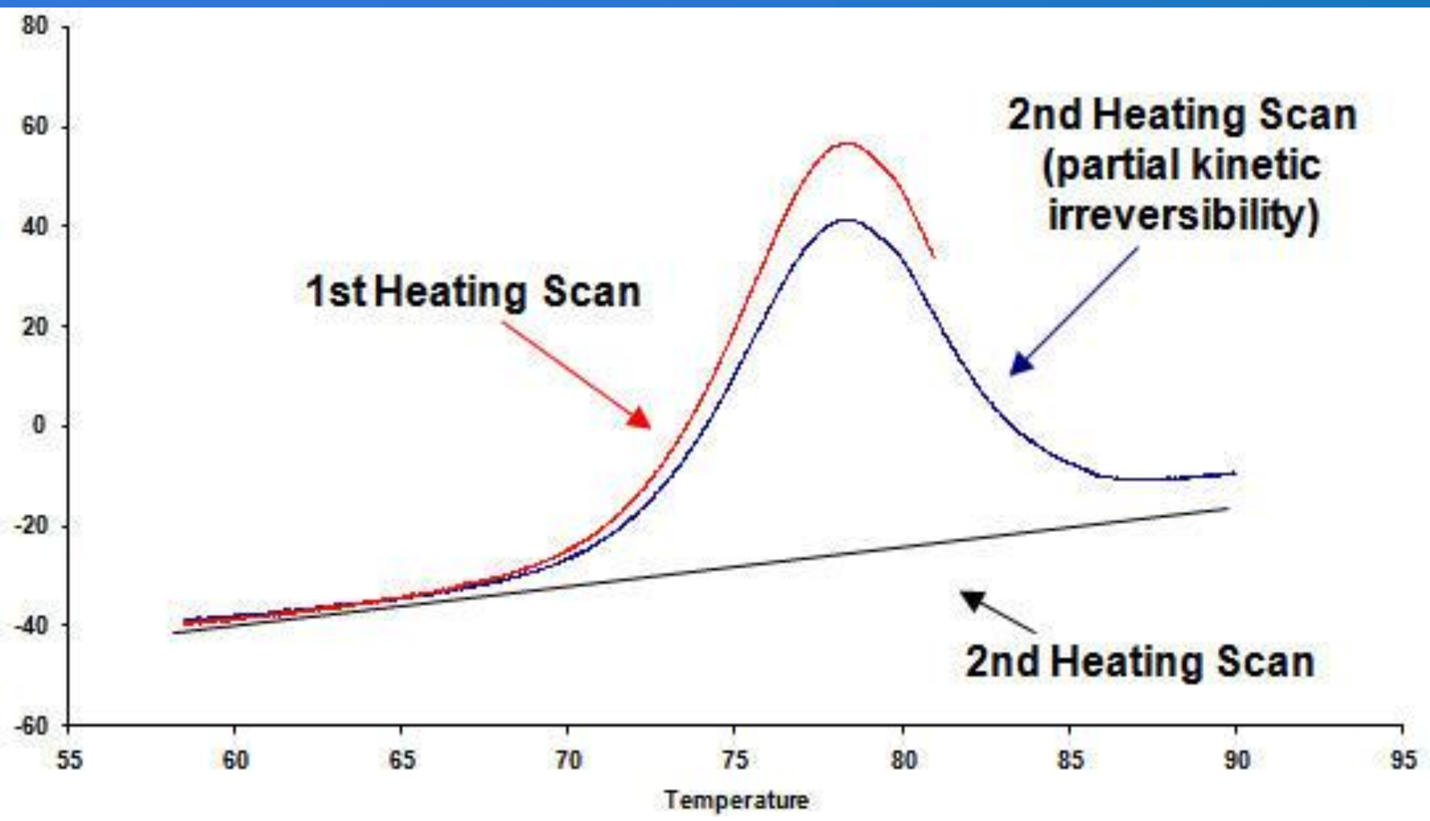
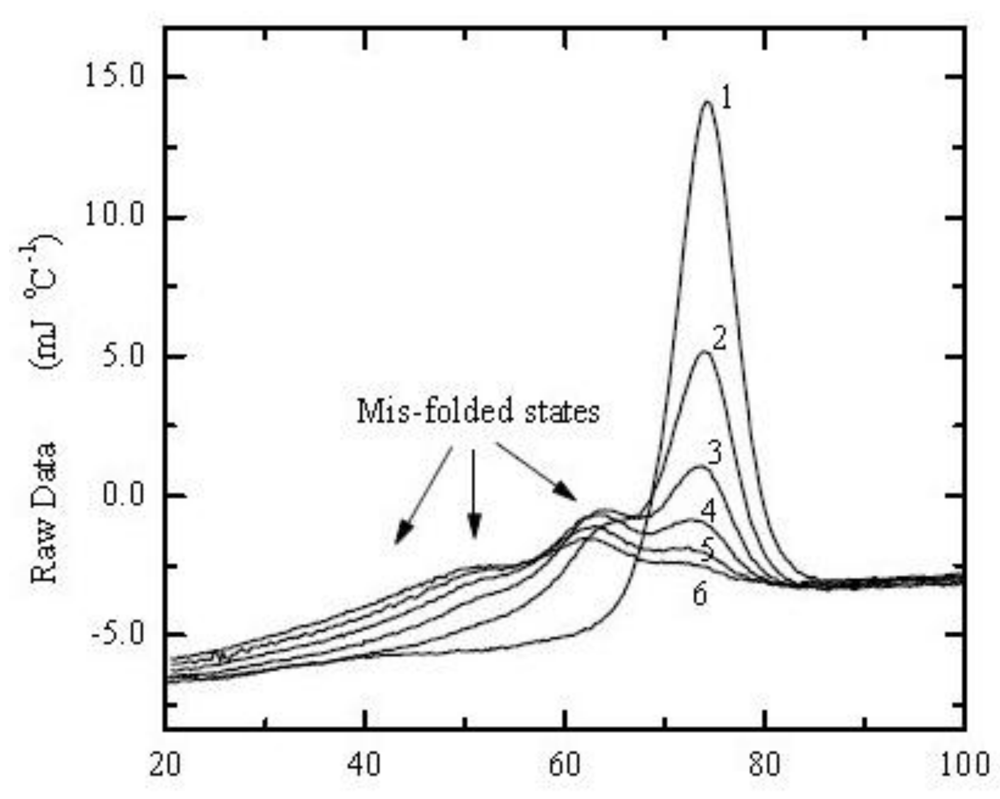
How to determine if unfolding is reversible?



- Aggregation is characterized by thermodynamic and kinetic components.
  - Thermodynamic component causes unfolding.
  - Kinetic component can result in partial or complete irreversibility.

Protein unfolding/refolding reversibility - rescanning



1st heating scan to 6th heat scan shown

- Non-reversibility of protein unfolding indicates:
  - Multi-domain or subunit structure
  - Chemical alterations to the sequence
  - Kinetic events hindering

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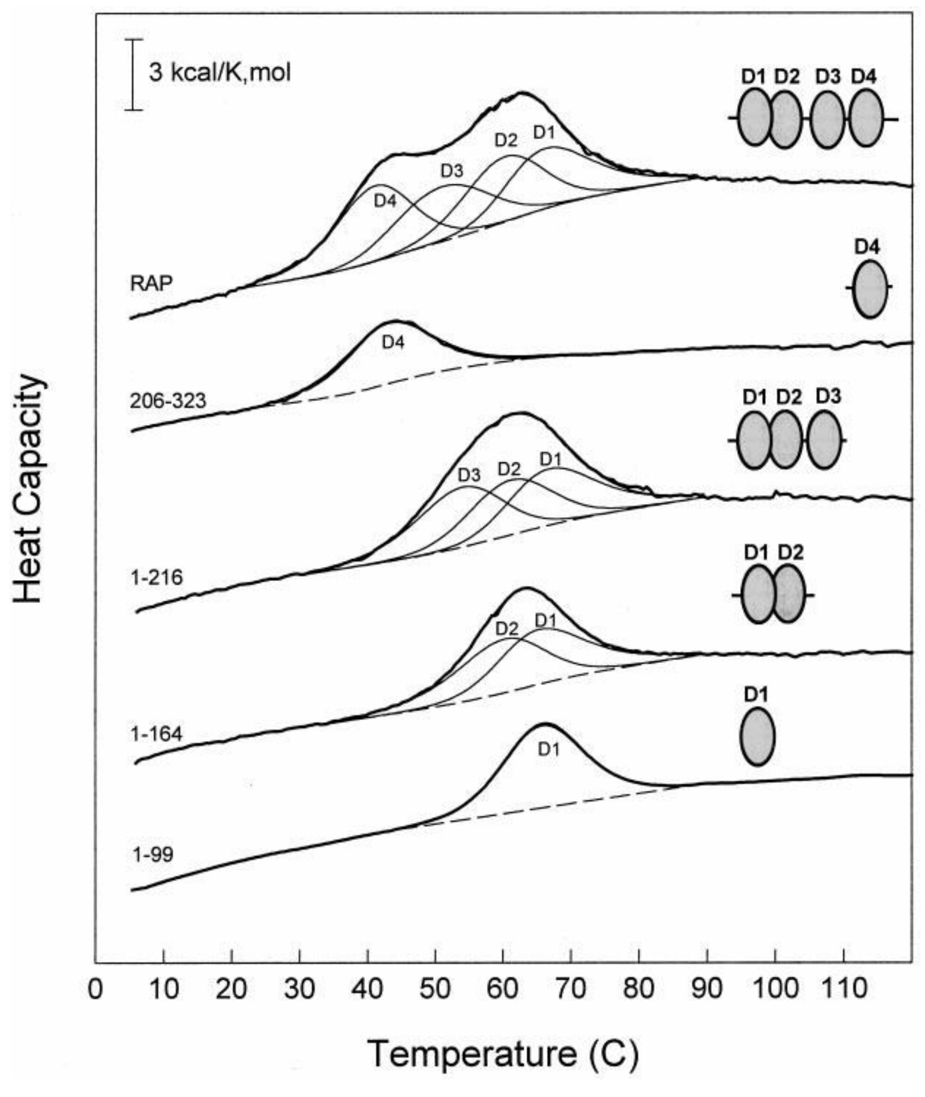
Domain and Subunit Stabilities

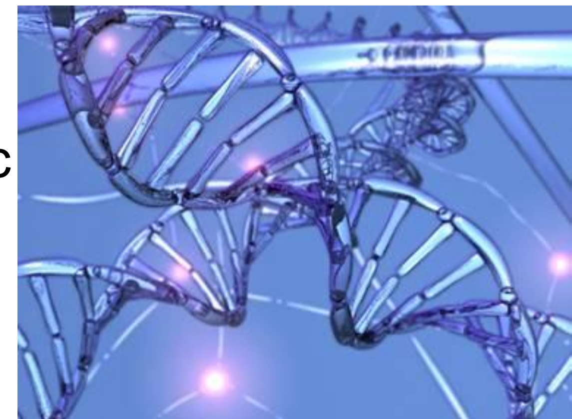


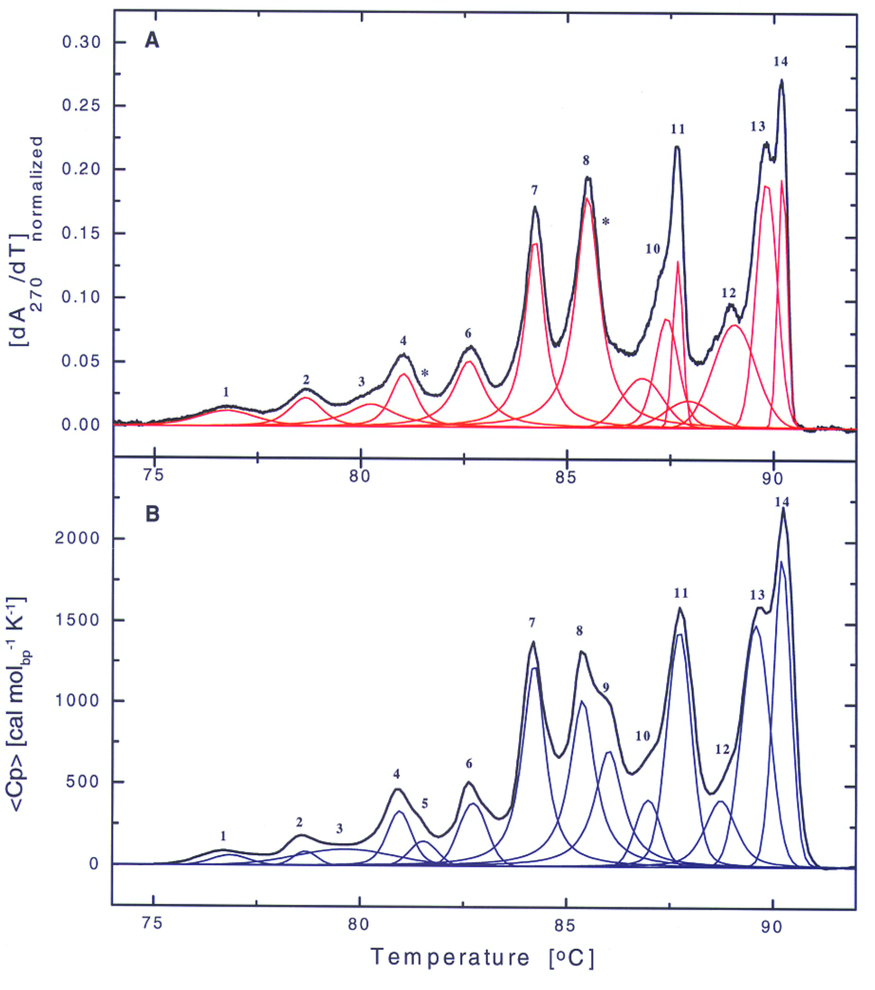
- Unfolding of domains and subunits with different thermal stabilities may produce asymmetric thermograms.
- A small change in sequence, or other alteration, can affect the stability of the whole protein, or the stability of one domain or subunit.
- DSC quickly reveals these stability changes.Practical implications: Identify subunit stability within a protein.

Medved *et al*., *J. Biol. Chem*. **274**, 717-727, 1999

Polynucleotide structure

- Linearlized pBR 322 heated at 0.1 ^{o}C/min for optical profile, 1 ^{o}C/min for DSC scan.
- Individual spectral peaks reflect the cooperative melting of one or more domains.
- DSC provides a complimentary and more direct means of measuring thermodynamic parameters of DNA.

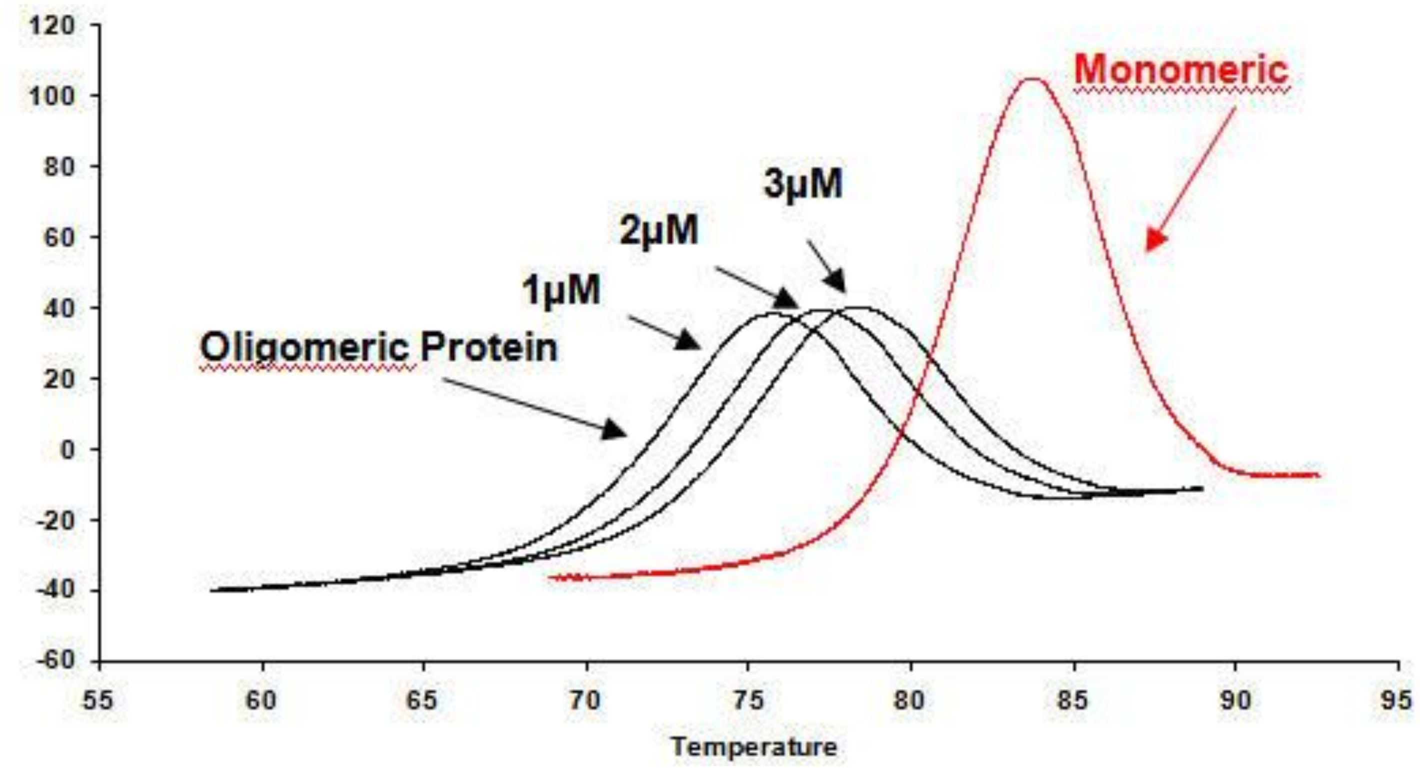




Volker *et al.*, *Biopolymers* **50**, 303-318, 1999

Importance of concentration determination

- Effect of sample concentration dependence of T_m is a test for oligomerization.



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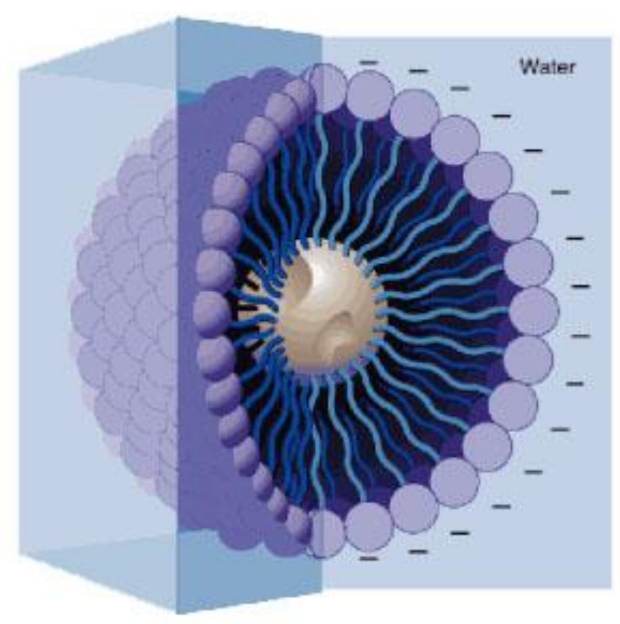
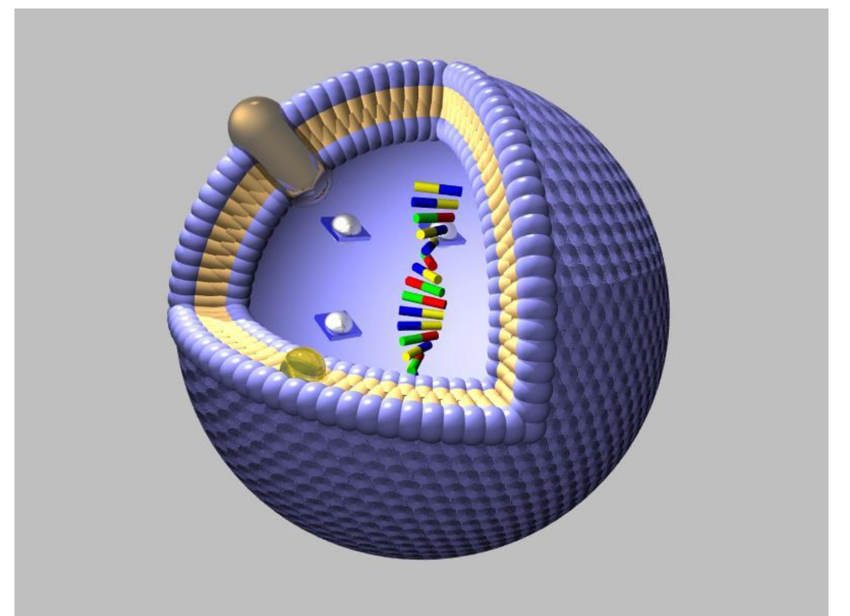
Membrane proteins and membranes: detergents and lipids

- Membrane proteins must be solubilized in detergents or lipids
- Detergents form nanospheres in water composed of a single layer of detergent molecules. This is called a micelle.
- Lipids form nanospheres in water composed of a double layer of lipid molecules, called a liposome.





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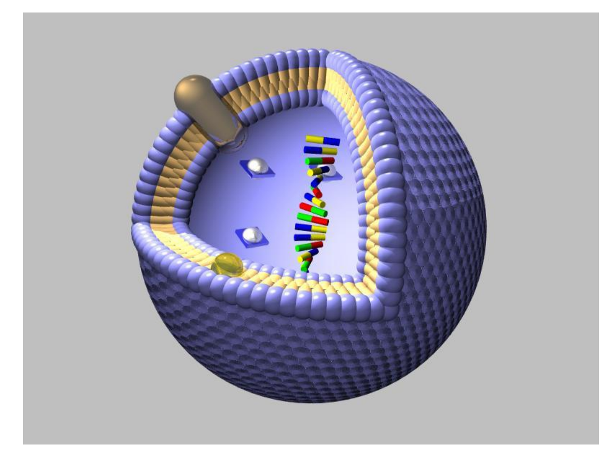
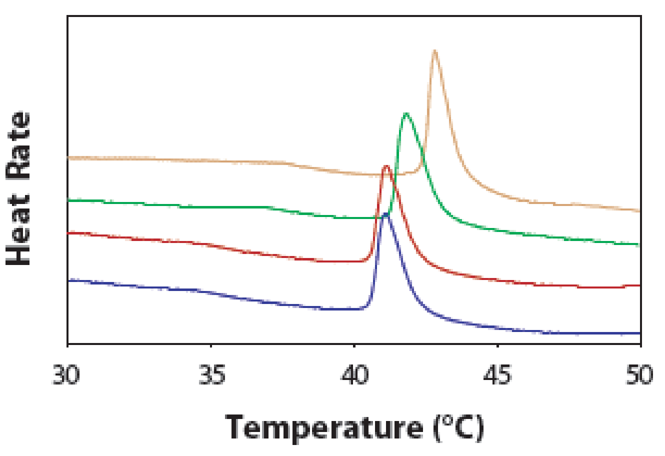
Membranes/Membrane protein Interactions

- Many proteins are associated with membranes and serve numerous functions.
- How does membrane stability change in the presence of various proteins
  - DSC can provide indications



- Vesicles were prepared by sonicaton bath with 5 mg/mL DPPC and then treated
  - Palmitoylated SNAP-25 (Orange)
  - 1.2 µg SNAP-25 (Red)
  - 2 µg SNAP-25 (Blue)
  - Untreated control (Green)



Palmitoylated SNAP-25 increase membrane stability, while non-palmitoylated SNAP-25 decreases membrane stability relative to the control

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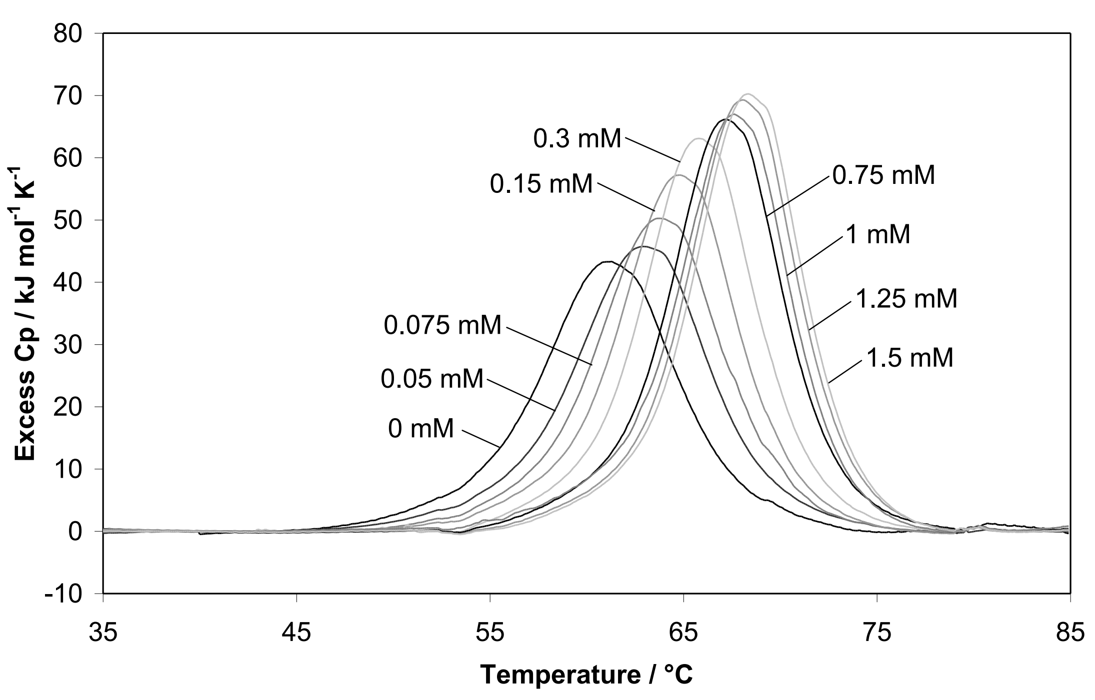
Studying Binding by DSC

- If a ligand binds preferentially to a folded protein, the T_m of the protein will generally increase. The more bound ligand there is, or the tighter it binds, the more T_m increases.
- Can determine binding constant at T_m.
- Useful for very tight binding
- DSC is a quick way to determine if two molecules interact.



Binding of 2′-CMP to RNase A ± 5% DMSO (black)
K_a = 5900 M^{-1} (-DMSO); 6900 M^{-1} (+DMSO) at T_m

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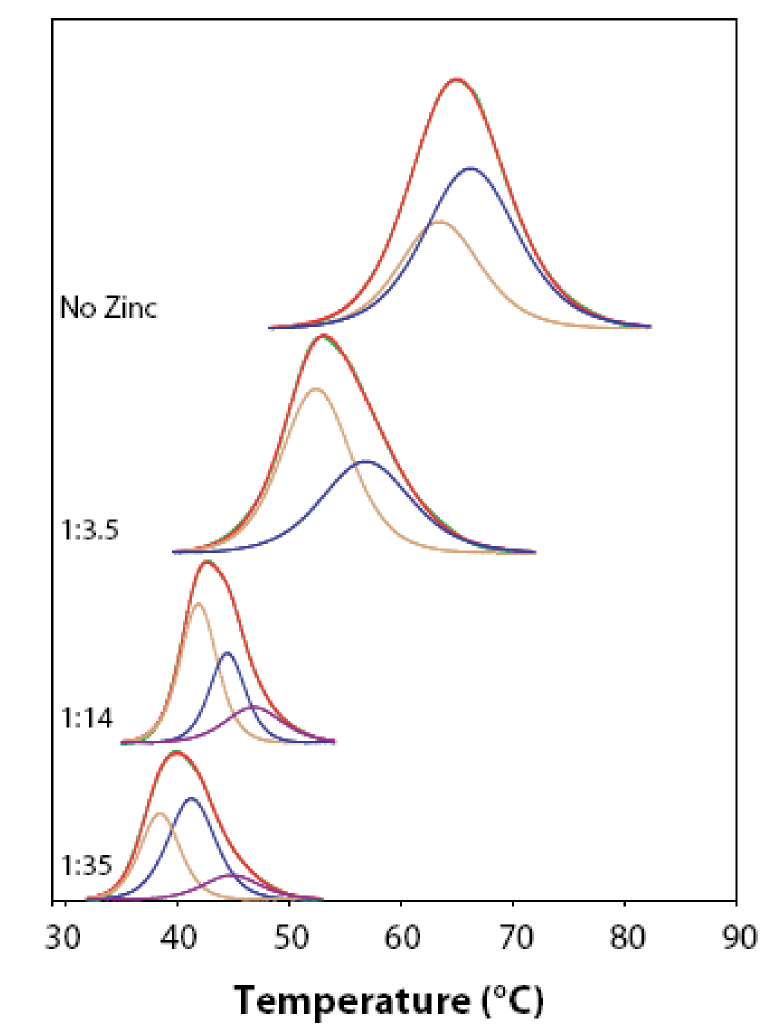
Tertiary structural changes upon ligand binding

- Protein structures are dynamic and constantly fluctuating between partially folding and folded structures
- Ligands can preferentially bind to a partially unfolded conformation
- Ex: Zn^{2+} added to Ca^{2+}-saturated a-lactalbumin, a two-domain protein
- Enthalpies of the domains in absence of Zn^{2+} are consistent with the crystal structure
- Increasing [Zn^{2+}] progressively destabilizes the protein, driving equilibrium towards the unfolded state
- DSC is a quick way of determining if a ligand binds preferentially to folded or partially unfolded protein.



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Thank You

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